ANNEX 2

Methods

^{1}H-NMR spectra of β-cyclodextrin (β-CD) complex was recorded in D_2O solution with a Varian spectrometer Mercury Plus (Varian Inc., Palo Alto, CA, USA) at 399.93 MHz. Chemical shifts are given in ppm (δ) which were measured relative to the peak of the solvent D_2O (4.65 ppm). All ^{1}H NMR spectra were recorded with a 5 mm tube in D_2O, without degassing.

^{1}H NMR spectroscopy has proved to be a good diagnostic tool as well as useful in the study and characterization of βCD inclusion complexes (references 1-3)

Because of the water solubility of all βCD-pyrethroid-PBO complexes at 20 °C at a reasonable concentration (>2 mM) is achieved, ^{1}H NMR experiments, performed in D_2O, provide further experimental evidence of the formation of these complexes since all pure pyrethroyd (i.e. not complexed) are far soluble in water as reported in Table 1.

Table 1. Solubility of the pyrethroids in water (mg/l)

α-cypermethrin	0.01mg/l
biphenthrin	0.1mg/l
λ-cyalothrin	0.005mg/l
β-cifluthrin	2mg/l

Protons of the pyrethroid included in the βCD cavity appear sharp and clear whereas no detectable proton signals of the pyrethroid are observable in a mechanical mixture with β-CD and PBO either when we tried to use our procedure to prepare a complex with βCD and pyrethroid without PBO. In our procedure, the presence of PBO allows to achieve a formulate βCD-pyrethroid-PBO. According to ^{1}H NMR data on β-cyclodextrin complexes reported in the literature (1,2,3), chemical shifts of H^3 and H^5 β-CD protons, which point into the lipophilic cavity, are a useful probe to observe formation of inclusion complexes and, hence, to evaluate the structural modification of β-CD.

Chemical shift variations of H^3 and H^5 β-CD protons reflect the formation of a complex between them. In fact, the entry of the apolar guest into the lipophilic cavity of the host (β-CD) induces a shielding of H^3 and H^5 as reported in Table 2. These data indicate that the pyretroids are included into the lipophilic cavity of β-CD.

Although PBO have structural and electronical features to form a complex included in the cavity of β-CD, all pyrethroids (pyrethroids) examined form a more stable complex with the β-CD cavity compared to PBO. However according our statement PBO that is present in this complex is hence reasonably located in the hydrophilic part of β-CD.

The release time of pyrethroid was calculated by ^1H NMR (recorded in D_2O) of the integration of the proton signals of the pyrethroid and β-CD.

Results

Table 2 shows the chemical shifts values for β-CD in the free and in the complex state measured in D_2O. The Δδ(ppm) represents the chemical shifts differences between the two states.

TABLE 2

	βCD Proton H3 (ppm)	*βCD Proton H5 (ppm)*	*H3 Δδ/H5 Δδ βCD free- βCD complex**	*Release time at 22 °C, start release*
βCD free	*3.822*	*3.715*	-------	
βCD –acip PBO complex	3.847	3.747	+0.025/+0.032	4 h
βCD – bipenthrin PBO complex	3.701	3.600	-0.120/-0.115	6h
βCD – λ-cyalothrin PBO complex	3.732	3.628	-0.020/-0.011	4h
βCD –βeifluthrin PBO complex	3.866	3.800	+0.044/+0.085	5h

* -Δδ: lower field; +Δδ: higher field

By analysis of NMR spectroscopic data, has been found that the pyrethroid begins to be delivered by the complex only after some hours; on the contrary more than 90% of PBO can be released by the complex very quickly when it is dissolved/suspended in water, thereby maximising the synergystic effect.

In fact, it is interesting to observe in the ^1H NMR spectrum of formulate (recorded in D_2O) the presence of the same pattern of PBO protons (PBO *state A*) shifted to upfield (PBO *state B*) (Table 3). The amount of PBO *state B* is about 10% respect to PBO *state A*. It means that when the complex of the present invention is suspended in water more than 90% of PBO is immediately released, realizing our goal to introduce the better Δt (>4h) between the release of the two components (pyretroid and synergic).

Table 3. Chemical shifts values in ppm for PBO in the state A and in the state B measured in D2O

TABLE 3

	$OCH_2O_{state\ A}$ (proton, multiplicity)	OCH_2O (proton, multiplicity)	$OCH_2O_{state\ B}$ ppm	$\Delta\delta$ $OCH_2O_{state\ B}$ and $OCH_2O_{state\ A}$
PBO Free	----------	5.465 (2H, s)	-------	-----
BCD -acip PBO complex	5.485 (2H, s)	----------	5.769 (2H, s)	+0.284
BCD – biphenthrin PBO complex	5.360 (2H, s)	----------	5.660 (2H, s)	+0.300
BCD – λ-cyalothrin PBO complex	5.462 (2H, s)	----------	5.750 (2H, s)	+0.288
BCD –βcifluthrin PBO complex	5.449 (2H, s)	----------	5.750 (2H, s)	+0.201

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